Pdx1 restores β cell function in Irs2 knockout mice

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The homeodomain transcription factor Pdx1 is required for pancreas development, including the differentiation and function of β cells. Mutations in Pdx1 or upstream hepatocyte nuclear factors cause autosomal forms of early-onset diabetes (maturity-onset diabetes of the young [MODY]). In mice, the Irs2 branch of the insulin/Igf signaling system mediates peripheral insulin action and pancreatic β cell growth and function. To investigate whether β cell failure in *Irs2*$^{-/-}$ mice might be related to dysfunction of MODY-related transcription factors, we measured the expression of Pdx1 in islets from young *Irs2*$^{-/-}$ mice. Before the onset of diabetes, Pdx1 was reduced in islets from *Irs2*$^{-/-}$ mice, whereas it was expressed normally in islets from wild-type or *Irs1*$^{-/-}$ mice, which do not develop diabetes. Whereas male *Irs2*$^{-/-}$*Pdx1*$^{+/+}$ mice developed diabetes between 8 and 10 weeks of age, haploinsufficiency for *Pdx1* caused diabetes in newborn *Irs2*$^{-/-}$ mice. By contrast, transgenic expression of Pdx1 restored β cell mass and function in *Irs2*$^{-/-}$ mice and promoted glucose tolerance throughout life, as these mice survived for at least 20 months without diabetes. Our results suggest that dysregulation of Pdx1 might represent a common link between ordinary type 2 diabetes and MODY.

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Introduction

The insulin receptor substrates (IRS proteins) coordinate many signals during insulin and IGF-1 stimulation, including activation of the phosphatidylinositol (PI) 3-kinase and ERK1/2 cascades (1). Although highly homologous and universally expressed, Irs1 and Irs2 display distinct biological function in mice (2). Irs1 strongly promotes somatic growth and mediates insulin action upon carbohydrate metabolism in skeletal muscle; however, *Irs1*$^{-/-}$ mice never develop diabetes, owing to islet hyperplasia and lifelong compensatory hyperinsulinemia (2). By contrast, Irs2 plays a significant role in metabolic regulation, as hepatic gluconeogenesis and lipid metabolism are dysregulated in *Irs2*$^{-/-}$ mice (3). Moreover, *Irs2*$^{-/-}$ mice develop progressive β cell failure, which exacerbates the peripheral insulin resistance and leads to diabetes (2). The β cells in *Irs2*$^{-/-}$ mice display increased apoptosis, especially during weaning, which might contribute to the steady decline of β cell mass with increasing age (4). Although moderate hyperinsulinemia compensates for peripheral insulin resistance in young *Irs2*$^{-/-}$ mice, diabetes occurs between 8 and 10 weeks of age, because the diminishing β cell content fails to secrete sufficient insulin (2).

Autosomal dominant forms of early-onset diabetes in young adults (maturity-onset diabetes of the young [MODY]) are linked to mutations in glucokinase or transcription factors that promote normal β cell function, including *HNF4α* (MODY1), *HNF1α* (MODY3), *PDX1* (MODY4), *HNF1β* (MODY5), and *NeuroD1/BETA2* (MODY6); MODY2 is caused by mutations in *glucokinase*, whose expression is regulated by PDX1 (5). The regulation of these transcription factors is complicated, but in certain cases it might be linked to insulin/IGF1 signaling though *Foxo1* or *Hnf3β* (6). Pdx1 plays an important role in islet development and β cell function. It is required for pancreas formation during the initial stages of gut development (7, 8). In adult mice, Pdx1 promotes the expression of proinsulin, *Glut2*, and *glucokinase*, which mediates glucose-sensitive insulin secretion (9–11). Pdx1 also regulates expression of FGFs and their receptors, which might promote β cell growth and proinsulin processing via the prohormone processing enzyme PC1/3 (12). Consequently, disruption of *Pdx1* in murine β cells reduces insulin secretion and causes progressive β cell loss, which culminates in glucose intolerance and diabetes (13). Moreover, *PDX1* is required in adult humans to promote normal glucose sensing and insulin secretion, and mutations in *PDX1* represent a risk factor for type 2 diabetes (13–15). In this report, we reveal a close relation between Pdx1 and Irs2, which suggests that dysregulation of Pdx1 by genetic or functional mechanisms might be a com-

mon element in autosomal early-onset (MODY) and common type 2 diabetes.

Methods

Mice. *Irs1* knockout (2), *Irs2* knockout (2), *Pdx1* knockout (8), and wild-type *Pdx1* transgene (16) mice were maintained on a mixed C57BL/6 × 129Sv × Black Swiss background. Genotyping of animals was done by PCR as previously described (4, 16). Male mice were used to characterize adult glucose homeostasis phenotypes and pancreatic pathology. Male and female $Irs2^{+/-}Pdx1^{+/-}$ intercross newborn pups were grouped together, as no sexual dimorphism was apparent in neonatal glucose homeostasis (data not shown). Random-fed glucose and insulin measurements were performed as previously described (4). Intraperitoneal glucose tolerance tests were performed on mice fasted for 15–16 hours with 2 g D-glucose per kg body weight as previously described (2). Intraperitoneal insulin tolerance tests were performed on fed mice with 0.75 U human insulin per kg body weight as previously described (2).

Immunohistochemistry. Immunohistochemical localization of antigens and double-label immunohistochemistry were performed similarly to previously described methods (4). Pancreas samples were dissected from fed mice and fixed with Bouin's solution overnight. Five-micrometer longitudinal sections of paraffin blocks were rehydrated with xylene followed by decreasing concentrations of ethanol, microwaved in 0.01 M sodium citrate (pH 6.0) for 20 minutes, and permeabilized with 1% Triton X-100 in PBS prior to primary antisera incubation. Islet morphometry was performed similarly to the methods previously described (4). The primary antibodies were: guinea pig anti-insulin and rabbit anti-glucagon antibodies (Zymed Laboratories Inc., South San Francisco, California, USA) rabbit N-terminal anti-*Pdx1* (from C.V.E. Wright), and mouse anti-BrdU (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Secondary antibodies were labeled with FITC or rhodamine (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA).

Islet morphometry. Newborn (postnatal day [P] 0.5–1.5) β cell area was quantified by acquiring adjacent ×10 images of the entire newborn pancreas from two anti-insulin–stained sections per animal, at least six animals per genotype (male and female were grouped together), with a Zeiss Axiovert microscope (Carl Zeiss MicroImaging, Thornwood, New York, USA). Images were analyzed for area with Improvision Open Lab software density slice software (Improvision Scientific Imaging, Lexington, Massachusetts, USA). For each animal the ratio of β cell area to total pancreas area was calculated and reported as mean ± SEM. Adult β cell area was measured by acquiring images from two sets of eight to ten random nonoverlapping images at ×10 of insulin- and glucagon-stained sections from male mice, at least four animals per genotype. Results of β cell quantification are expressed as the percentage of the total surveyed area containing cells positive for insulin. Islet density and size were calculated from captured insulin-stained images. Ratios of β to α cells were calculated from mean insulin- and glucagon-positive cell areas. Islet proliferation and β cell size were examined by injecting 2- to 3-week-old male mice with 5-bromo-2-deoxyuridine (BrdU; Roche Molecular Biochemicals, Indianapolis, Indiana, USA; 100 μg/g body weight) and performing double-label insulin and BrdU immunohistochemistry on rehydrated Bouin's-fixed paraffin-embedded sections from the mice. Images of each islet per section were acquired at ×63. BrdU-positive β cell ratios were calculated as the mean ± SEM of BrdU-positive β cells over total β cells per section, two sections per animal, three to four animals per genotype. Size of β cells was determined by dividing total β cell area by the number of β cells examined per section.

RT-PCR and Western blotting. Islets were isolated from 6-week-old male wild-type, $Irs1^{-/-}$, and $Irs2^{-/-}$ mice with Collagenase P (Liberase HI; Roche Molecular Biochemicals) digestion (17) and total RNA was extracted with Trizol (GIBCO BRL; Life Technologies Inc., Grand Island, New York, USA) and RNeasy (QIAGEN Inc., Valencia, California, USA) columns. cDNA synthesis was performed using the RETROscript kit (Ambion Inc., Austin, Texas, USA) and then TaqMan (Applied Biosystems, Foster City, California, USA) quantitative PCR (50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute) was performed with the ABI Prism 7700 PCR instrument (Applied Biosystems) to amplify samples in triplicate for the *Pdx1*, *Hnf1α*, *Hnf1β*, *Hnf3β* (*Foxa2*), *Hnf4α*, and *cyclophillin* genes. Primers were as follows: Pdx1 forward, AGGAAAACAAGAGGACCCGTACT; Pdx1 probe, CCTACACCCGGGCGCAGCTG; Pdx1 reverse, CGGGAGATGTATTTGTTAAATAAGAATTC; Hnf1α forward, TGCGTTCTACAACTGGTTTGC; Hnf1α probe, AGGCTTCCTCCTTGCGCCGG; Hnf1α reverse, GGAGGTCCGTTATAGGTGTCCAT; Hnf1β forward, GAGGAGTGTAACAGGGCAGAATGT; Hnf1β probe, CCTTCCAAAGCCCACGGCCTAGG; Hnf1β reverse, GGACCTCCGTGACCAAGTTG; Hnf3β forward, GAACTCCATCCGCCACTCTCT; Hnf3β probe, TCAACGACTGCTTTCTCAAGGTGCCC; Hnf3β reverse, GCCCTTGCCAGGCTTGT; Hnf4α forward, ACGTGCTGCTCCTAGGCAAT; Hnf4α probe, CACACGGCTCATCTCCGCTAGCTCTG; Hnf4α reverse, TCGAGGATGCGGATGGA; cyclophillin forward, CAGACGCCACTGTCGCTTT; cyclophillin probe, CCTACACCCGGGCGCAGCTG; cyclophillin reverse, TGTCTTTGGAACTTTGTCTGCAA. For each sample, triplicate gene expression values were compared with wild-type islet cDNA dilution standard curves of $\log_4$ dilutions (undiluted, 1:4, 1:16, 1:64) performed in triplicate (*Pdx1* threshold cycle (C_t) = 23, r^2 = 0.99; Hnf1α C_t = 28, r^2 = 0.96; Hnf1β C_t = 29, r^2 = 0.88; Hnf3β C_t = 27, r^2 = 0.95; Hnf4α C_t = 29, r^2 = 0.99; cyclophillin C_t = 21, r^2 = 0.98). Relative gene product amounts were reported as mean ± SEM of several animals for each gene compared with cyclophillin (wild-type, n = 4; $Irs1^{-/-}$, n = 2; $Irs2^{-/-}$, n = 5). For

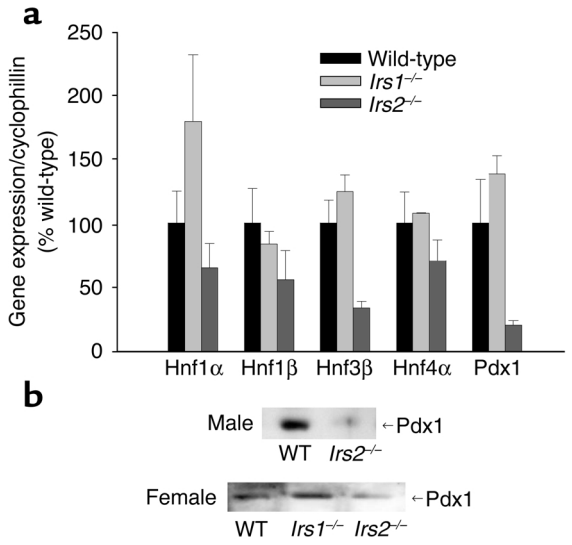

Figure 1
Expression of hepatocyte nuclear factors (HNFs) and *Pdx1* in pancreatic islets. (**a**) TaqMan RT-PCR of Hnf1α, Hnf1β, Hnf3β, Hnf4α, and Pdx1 in islets from wild-type (WT) (n = 4), *Irs1*$^{-/-}$ (n = 2), and *Irs2*$^{-/-}$ (n = 5) male mice at 6 weeks of age. Data are normalized to cyclophillin gene expression and expressed as mean ± SEM. (**b**) Western blots of Pdx1 in islets from male (top) and female (bottom) mice.

Western blot analysis, SDS protein islet lysates were equalized for loading by total protein assay (Pierce Chemical Co., Rockford, Illinois, USA) run on 12% SDS-PAGE, transferred to a Protan nitrocellulose membrane (Schleicher & Schuell Inc., Keene, New Hampshire, USA), incubated with rabbit anti–N-terminal Pdx1 antisera (from C.V.E. Wright) and HRP-conjugated goat anti-rabbit antisera, and then treated for enhanced chemiluminescence (NEN Life Science Products Inc., Boston, Massachusetts, USA).

Results

Pdx1 mRNA levels were measured by RT-PCR in islet extracts from young male mice. Compared with wild-type, Pdx1 mRNA levels were reduced in *Irs2*$^{-/-}$ islets (Figure 1a); similar results were found with age-matched female *Irs2*$^{-/-}$ mice (data not shown). Hnf3β (also known as Foxa2) was also reduced in islets from *Irs2*$^{-/-}$ mice, whereas the levels of Hnf1α, Hnf1β, and Hnf4α were approximately normal in the male *Irs2*$^{-/-}$ mice (Figure 1a). Immunoblotting confirmed that Pdx1 protein levels were low in male and female *Irs2*$^{-/-}$ islets (Figure 1b). By contrast, mRNA levels for Pdx1 and the various hepatocyte nuclear factors were nearly normal in *Irs1*$^{-/-}$ islets at 6 weeks of age, and immunoblotting confirmed that Pdx1 protein was normal in female *Irs1*$^{-/-}$ mice (Figure 1b). The specific reduction of Pdx1 expression in *Irs2*$^{-/-}$ mice might be especially important for the progression to diabetes, as Pdx1 regulates components of the glucose sensing pathway, and pathological processes that reduce *Pdx1* expression cause glucose intolerance (11, 15).

To test the role of Pdx1 in *Irs2*$^{-/-}$ mice, we intercrossed *Irs2*$^{+/-}$ mice with *Pdx1*$^{+/-}$ mice and backcrossed the compound heterozygotes to produce the nine expected genotypes at mendelian frequencies (8). On P2.5, haploinsufficiency for *Pdx1* or *Irs2* had no effect on body weight, whereas *Irs2*$^{-/-}$ mice were slightly smaller by comparison with littermates (Figure 2a). *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ mice and mice lacking *Pdx1* (*Pdx1*$^{-/-}$, *Irs2*$^{+/-}$*Pdx1*$^{-/-}$, or *Irs2*$^{-/-}$*Pdx1*$^{-/-}$) were about 40% smaller at P2.5 and failed to gain weight thereafter (Figure 2a and data not shown). During the first 5 postnatal days, most mice displayed normal blood glucose, including the *Irs2*$^{-/-}$ and *Pdx1*$^{-/-}$ mice (Figure 2b). However, *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ mice developed severe hyperglycemia and died by P4; *Irs2*$^{-/-}$*Pdx1*$^{-/-}$ mice displayed a similar outcome (Figure 2c and data not shown). No sexual dimorphism was seen in the *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ mouse phenotypes, as both male (n = 5) and female (n = 10) *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ pups developed hyperglycemia and died by P4. By contrast, *Pdx1*$^{-/-}$ mice displayed normal blood glucose at P2.5 and survived for up to 9 days, suggesting that Irs2 signaling might promote neonatal glucose homeostasis without insulin (Figure 2b). As shown previously, *Irs2*$^{-/-}$ mice

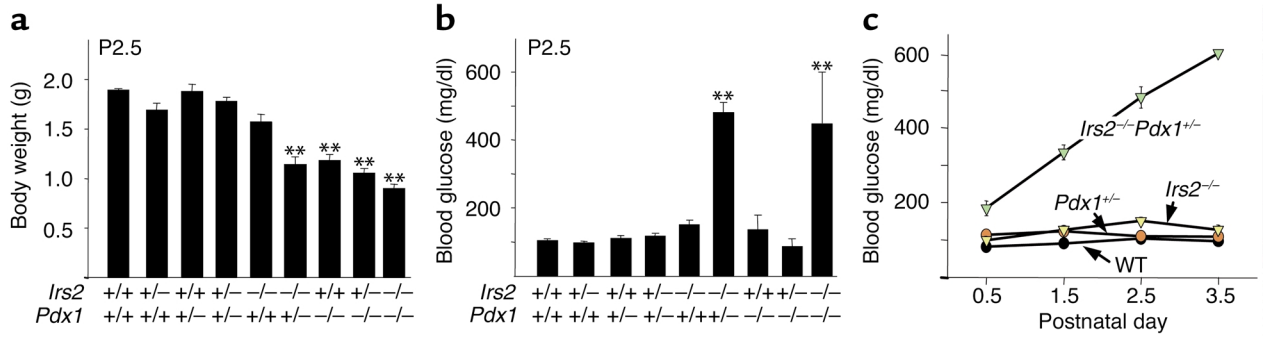

Figure 2
Characterization of progeny of *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ intercross. (**a**) Body weights of *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ intercross pups measured on P2.5. Results are reported as mean ± SEM of at least seven mice (except *Irs2*$^{-/-}$*Pdx1*$^{-/-}$ mice, n = 3). $^{**}P < 0.01$ compared with all other genotypes. (**b**) Blood glucose of *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ intercross pups measured on P2.5. Results are reported as mean ± SEM of at least seven mice, or three *Irs2*$^{-/-}$*Pdx1*$^{-/-}$ mice. $^{**}P < 0.01$ for *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ or *Irs2*$^{-/-}$*Pdx1*$^{-/-}$ mice compared with all other genotypes. (**c**) Blood glucose values of wild-type, *Irs2*$^{-/-}$, *Pdx1*$^{+/-}$, and *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ mouse pups in the first 4 days of life. Results are reported as mean ± SEM of at least seven mice.

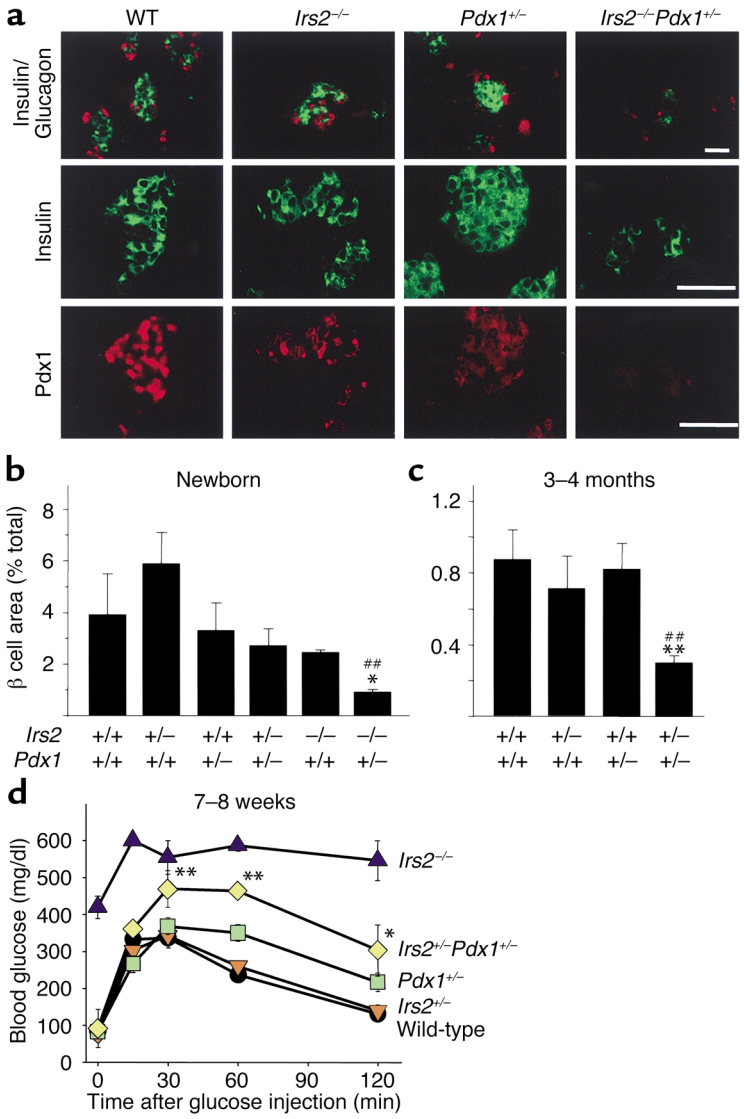

Figure 3
Islet morphology and quantification from *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ intercross mice and glucose tolerance tests of adult *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ intercross mice. (**a**) Representative islet morphology from pancreas of newborn *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ intercross pups immunostained with antibodies against insulin (green) and glucagon (red, top panels), insulin (green, middle panels), and Pdx1 (red, bottom panels). Scale bars, 50 μm. (**b**) β Cell area of newborn male and female progeny from *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ intercross pups (mean ± SEM relative to total pancreas area). *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ pups have decreased β cell area compared with wild-type, *Irs2*$^{-/-}$, or *Pdx1*$^{+/-}$ (*P < 0.05, ##P < 0.01). (**c**) Morphometric analysis of pancreas sections of 3- to 4-month-old male progeny from the *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ intercross. Results are reported as mean ± SEM of β cell area (percent relative to total pancreas area). At 3–4 months, *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ mice have decreased β cell area compared with wild-type (**P < 0.01) or *Pdx1*$^{+/-}$ (##P < 0.01). (**d**) Glucose tolerance tests of 7- to 8-week-old mice performed with 2 g D-glucose per kg body weight after a 15- to 16-hour fast (wild-type, n = 6; *Irs2*$^{+/-}$, n = 11; *Pdx1*$^{+/-}$, n = 9; *Irs2*$^{+/-}$*Pdx1*$^{+/-}$, n = 11; *Irs2*$^{+/-}$*Pdx1*$^{+/-}$, n = 17; *Irs2*$^{-/-}$, n = 4). Results are reported as mean ± SEM. *P < 0.05, *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ vs. *Pdx1*$^{+/-}$; **P < 0.01, *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ vs. *Pdx1*$^{+/-}$.

developed fasting hyperglycemia at 8 weeks and died between 12 and 15 weeks of age (2, 7).

Pancreas sections were immunostained with antibodies against insulin and glucagon to characterize islet histology and estimate pancreatic β cell content. As expected, the pancreas failed to develop in mice lacking *Pdx1*, including *Irs2*$^{+/-}$*Pdx1*$^{-/-}$ and *Irs2*$^{-/-}$*Pdx1*$^{-/-}$ mice; however, it developed in proportion to body size in the other mice (data not shown). At birth, *Irs2*$^{-/-}$*Pdx*$^{+/-}$ islets displayed few and small insulin-positive β cell clusters, compared with the normal-sized islets in *Irs2*$^{-/-}$, *Pdx1*$^{+/-}$, and wild-type mice (Figure 3a). Pdx1 immunostaining was readily detected in wild-type islets but was consistently reduced in *Pdx1*$^{+/-}$ and in *Irs2*$^{-/-}$ islets; and it was nearly undetected in *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ islets, consistent with the severe hyperglycemia that developed at birth (Figure 3a).

β Cell content was quantified in the various mice by estimating the insulin-positive area in multiple pancreatic sections. Compared with wild-type mice, β cell content was slightly increased in newborn *Irs2*$^{+/-}$ mice and slightly decreased in *Pdx1*$^{+/-}$, *Pdx1*$^{+/-}$*Irs2*$^{+/-}$, and *Irs2*$^{-/-}$ mice; however, these small changes did not reach statistical significance (Figure 3b). By contrast, β cell content was significantly reduced in pancreatic slices from newborn *Irs2*$^{-/-}$*Pdx*$^{+/-}$ mice (P < 0.05), consistent with the onset of fatal hyperglycemia 4 days after birth (Figure 3b). By 3–4 months of age, β cell content was similar in *Irs2*$^{+/-}$, wild-type, and *Pdx1*$^{+/-}$ mice (Figure 3c); however, β cell content was reduced by 75% in compound heterozygous *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ mice, suggesting that both Irs2 and Pdx1 contribute to normal islet function (Figure 3c). Consequently, *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ mice displayed more severe glucose intolerance than did less affected littermates at 7–8 weeks of age (Figure 3d).

To test whether Pdx1 promotes normal function of Irs2$^{-/-}$ β cells, we crossed *Irs2*$^{+/-}$ mice with *Pdx1*^{tg} mice to increase the *Pdx1* gene dosage in the *Irs2*$^{-/-}$ mice. As previously described, *Pdx1*^{tg} mice have multiple copies of a rat *Pdx1* cDNA controlled by the rat *Pdx1* promoter, which results in approximately threefold Pdx1 overex-

pression in the appropriate locations and developmental stages (16). Random-fed glucose and insulin levels were identical in wild-type and *Pdx1*^{tg} mice (Figure 4, a and b). Remarkably, *Pdx1*^{tg} expression prevented the hyperglycemia and relative hypoinsulinemia that developed in *Irs2*$^{-/-}$ mice during the first 12–13 weeks of life (Figure 4, a and b). Moreover, *Irs2*$^{-/-}$*Pdx1*^{tg} mice survived with nearly normal glucose and insulin levels for up to 16 months; we now have *Irs2*$^{-/-}$*Pdx1*^{tg} mice that have survived with mild glucose intolerance for up to 20 months. Based on these results, the *Pdx1*^{tg} expression restored β cell function in *Irs2*$^{-/-}$ mice and prevented the development of diabetes.

Although *Pdx1*^{tg} expression promoted β cell function in *Irs2*$^{-/-}$ mice, it had no significant effect on the sensitivity of wild-type mice to exogenous insulin injections and only slightly restored responsiveness of *Irs2*$^{-/-}$*Pdx1*^{tg} mice to insulin injections (Figure 4c). Glucose tolerance at 12–13 weeks of age was significantly improved in *Irs2*$^{-/-}$*Pdx1*^{tg} mice compared with *Irs2*$^{-/-}$ mice; however, tolerance was not entirely corrected, possibly owing to persistent peripheral insulin resistance (Figure 4d). *Irs2*$^{-/-}$ mice develop various disorders, including impaired brain and retinal growth, and infertility in females, and these defects persisted in *Irs2*$^{-/-}$*Pdx1*^{tg} mice, suggesting that they are a result of dysregulated *Irs2* signaling rather chronic hyperglycemia or diabetes (M. Schubert et al., manuscript submitted for publication; and ref. 18).

Transgenic expression of *Pdx1* had profound effects on the histology of *Irs2*$^{-/-}$ islets. At 4–5 weeks of age, immunostaining of insulin and Pdx1 in *Irs2*$^{-/-}$ islets was consistently reduced compared with that of wild-type mice (Figure 5). By contrast, insulin and Pdx1 immunostaining was consistently strong in *Pdx1*^{tg} and *Irs2*$^{-/-}$*Pdx1*^{tg} mice (Figure 5). Morphometric analysis of pancreatic sections at 4–5 weeks and at 3–4 months of age confirmed that β cell content (approximated by the mean cross-sectional β cell area) was significantly reduced in *Irs2*$^{-/-}$ mice (Table 1). *Pdx1*^{tg} expression did not change β cell content in wild-type pancreas sections, whereas it restored β cell content in the *Irs2*$^{-/-}$*Pdx1*^{tg} sections (Table 1). Moreover, *Pdx1*^{tg} expression increased the β cell/α cell ratio in wild-type and *Irs2*$^{-/-}$ mice, especially at 3–4 months of age (Table 1). Although β cell content in *Irs2*$^{-/-}$ sections decreased with age, the number of islets detected was barely reduced, and *Pdx1*^{tg} expression preserved or slightly increased the number of islets detected in the pancreas sections (Table 1). Thus, *Pdx1*^{tg} expression largely restores islet morphology in *Irs2*$^{-/-}$ mice.

Since the size of β cells was barely affected by the absence of *Irs2* or the presence of *Pdx1*^{tg} (Table 1), the increased β cell content in *Pdx1*^{tg} mice pancreas might be related, at least in part, to increased mitogenesis. Mitogenesis was estimated by the incorporation during 6 hours of BrdU into β cells of 2- to 3-week-old male mice. BrdU labeling of *Irs2*$^{-/-}$ β cells was decreased about 40% compared with wild-type mice, consistent with decreased β cell content. By contrast, BrdU labeling was increased twofold in both *Pdx1*^{tg} and *Irs2*$^{-/-}$*Pdx1*^{tg} mice (Figure 6). Thus, Pdx1-mediated mitogenesis might contribute to increased β cell content of *Irs2*$^{-/-}$ mice.

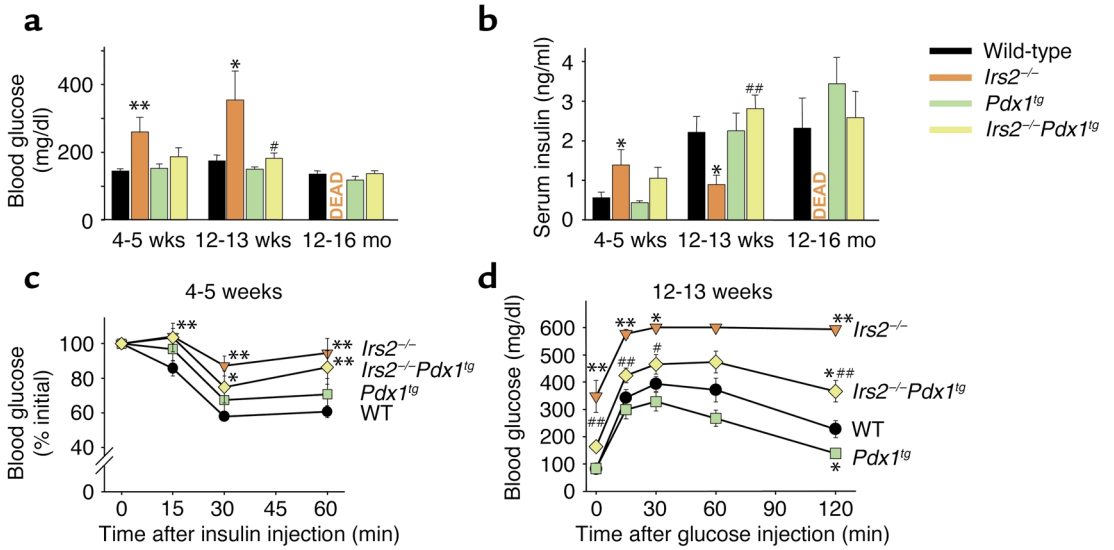

Figure 4
Characterization of male progeny of *Irs2*$^{+/-}$*Pdx1*^{tg} intercross. (**a** and **b**) Random-fed blood glucose (**a**) and serum insulin (**b**) of *Irs2*$^{+/-}$*Pdx1*^{tg} intercross mice at 4–5 weeks, 12–13 weeks, and 12–16 months. Results are mean ± SEM of six mice per genotype. *P < 0.05, *Irs2*$^{-/-}$ vs. wild-type; **P < 0.01, *Irs2*$^{-/-}$ vs. wild-type; $^{\#}P$ < 0.05, *Irs2*$^{-/-}$*Pdx1*^{tg} vs. *Irs2*$^{-/-}$; $^{\#\#}P$ < 0.01, *Irs2*$^{-/-}$*Pdx1*^{tg} vs. *Irs2*$^{-/-}$. (**c**) Insulin tolerance test of 4- to 5-week-old fed mice performed with 0.75 U/kg human regular insulin on fed animals. Results are expressed as mean ± SEM of percent of initial blood glucose value for at least eight animals per genotype. *P < 0.05 and **P < 0.01 vs. wild-type. (**d**) Glucose tolerance tests of 12- to 13-week-old mice performed with 2 g D-glucose per kg body weight after a 15- to 16-hour fast. Results reported as mean ± SEM for at least eight animals per genotype. *P < 0.05 vs. wild-type; **P < 0.01 vs. wild-type; $^{\#\#}P$ < 0.01, *Irs2*$^{-/-}$*Pdx1*^{tg} vs. *Irs2*$^{-/-}$.

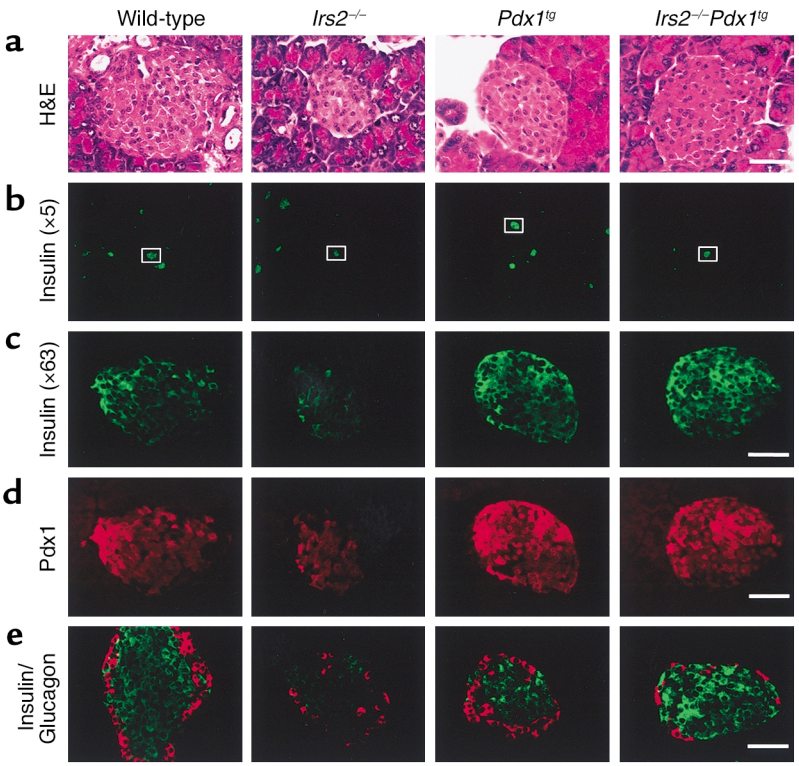

Figure 5
Islet morphology of male *Irs2*$^{+/-}$*Pdx1*^{tg} intercross mice. Representative pancreas sections from 4- to 5-week-old *Irs2*$^{+/-}$*Pdx1*^{tg} intercross mice. (**a**) Hematoxylin-and-eosin (H&E) staining at ×63. (**b** and **c**) Immunostaining with antibodies against insulin, photographed at ×5 (**b**) and ×63 (**c**). (**d**) Immunostaining with antibodies against Pdx1. (**e**) Immunostaining with antibodies against insulin (green) and glucagon (red). Scale bars, 50 μm.

Discussion

Our results show that the progressive loss of β cell function and the onset of diabetes in *Irs2*$^{-/-}$ mice are associated with decreased expression in pancreatic islets of the homeodomain transcription factor *Pdx1* (also called *Idx-1* and *Ipf1*). PDX1 is critical for the development of the pancreas in mice and people, and pancreas agenesis occurs upon the complete disruption of *PDX1* (7, 19). Moreover, PDX1 is required in adult humans and mice to promote normal glucose sensing and insulin secretion (13, 15). Genetic defects in the *PDX1* gene occur in about 5% of people with type 2 diabetes. Inactivating mutations are associated with autosomal early-onset diabetes (MODY), whereas missense mutations predispose humans to late-onset type 2 diabetes (14, 20).

The functional association between Irs2 signaling and Pdx1 expression observed in mice might relate type 2 diabetes to autosomal forms of diabetes (MODY). Genetic reduction of *Pdx1* in *Irs2*$^{-/-}$ mice causes diabetes at birth owing to severe reduction of islet β cell content. By contrast, transgenic expression of *Pdx1* postpones β cell failure and prevents the progression of *Irs2*$^{-/-}$ mice to diabetes. Partial reduction of both genes in *Irs2*$^{+/-}$*Pdx1*$^{+/-}$ mice causes an intermediate phenotype of severe glucose intolerance. If insulin resistance in people includes an IRS2 component, diminished IRS2 signaling might reduce PDX1 function and eventually impair β cell compensation, resulting in type 2 diabetes. Moreover, genetic defects in *PDX1* might exacerbate the effects of insulin resistance, even if the defects alone are physiologically silent until challenged by the need for a compensatory response. By contrast, people with robust PDX1 function might avoid diabetes even during chronic insulin resistance.

While mutations in *PDX1* are associated with human diabetes, naturally occurring *IRS2* mutations are very rare (21, 22). However, physiological stress associated with acute injury, chronic obesity, inactivity, or aging promotes peripheral insulin resistance. Recent evidence suggests that serine phosphorylation or regulated degradation of Irs proteins might contribute to this insulin resistance (23, 24). If functional dysregulation of IRS2 extends to β cells, the ability to compensate for peripheral insulin resistance by increasing β cell mass and insulin secretion might be impaired and lead to glucose intolerance and diabetes. A related mechanism could contribute to type 1 diabetes, where proinflammatory cytokines produced by infiltrating

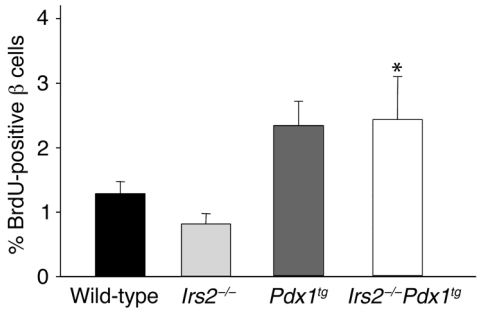

Figure 6
BrdU incorporation analysis of male *Irs2*$^{+/-}$*Pdx1*^{tg} intercross progeny at 2–3 weeks of age. Results are reported as percent BrdU-positive β cells per genotype, mean ± SEM. **P* < 0.05, *Irs2*$^{-/-}$*Pdx1*^{tg} vs. *Irs2*$^{-/-}$.

Table 1
Islet morphometry of *Irs2*$^{+/-}$*Pdx1*^{tg} intercross mice

Genotype	β Cell Area (% total)				Islet Size (μm² × 10³/islet)				Islet Density (islets/μm²)			
	4–5 weeks	*P*	3–4 months	*P*	4–5 weeks	*P*	3–4 months	*P*	4–5 weeks	*P*	3–4 months	*P*
Wild Type	0.51 ± 0.07		0.83 ± 0.19		3.1 ± 0.4		4.8 ± 0.5		1.3 ± 0.1		1.4 ± 0.1	
Irs2$^{-/-}$	0.26 ± 0.06	0.01	0.32 ± 0.04	0.01	2.5 ± 0.3	0.21	2.1 ± 0.2	< 0.01	0.8 ± 0.1	0.02	1.3 ± 0.1	0.22
Pdx1^{tg}	0.60 ± 0.06	0.38	1.30 ± 0.25	0.28	2.8 ± 0.3	0.4	3.9 ± 0.3	0.11	1.8 ± 0.1	0.02	2.8 ± 0.4	< 0.01
Irs2$^{-/-}$ *Pdx1*^{tg}	0.44 ± 0.05	0.04	0.80 ± 0.31	0.04	2.0 ± 0.2	0.2	4.6 ± 0.8	< 0.01	1.8 ± 0.3	0.01	1.4 ± 0.3	0.49

Genotype	β/α-Cell Ratio				β Cell Size (μm²/islet)	
	4–5 weeks	*P*	3–4 months	*P*	2–3 weeks	*P*
Wild Type	4.9 ± 0.6		17.2 ± 2.1		108 ± 6	
Irs2$^{-/-}$	4.1 ± 1.0	0.53	5.6 ± 1.8	< 0.01	96 ± 7	0.29
Pdx1^{tg}	8.5 ± 0.9	0.02	23.9 ± 2.2	0.07	107 ± 8	0.91
Irs2$^{-/-}$ *Pdx1*^{tg}	8.3 ± 1.3	0.03	28.2 ± 10.8	0.08	118 ± 3	0.03

Morphometric β cell area analysis of male *Irs2*$^{+/-}$*Pdx1*^{tg} intercross progeny at 4–5 weeks and 3 months of age. β Cell area was calculated from the mean cross-sectional β cell area of pancreas, reported as % of total pancreas area. Islet size was calculated from the mean cross-sectional β cell area per islet, reported as μm² × 10³/islet. Islet density was calculated from the mean islet density of pancreas, reported as islets/μm². β/α-Cell ratio was calculated as the mean cross-sectional β/α-cell area ratio. β Cell size was calculated from the mean cross-sectional β cell size, reported as μm²/islet. *Irs2*$^{-/-}$ *P* values against wild-type, *Pdx1*^{tg} *P* values against wild-type, *Irs2*$^{-/-}$*Pdx1*^{tg} *P* values against *Irs2*$^{-/-}$.

lymphocytes could inactivate Irs protein signaling and accelerate β cell death (25).

IRS-proteins coordinate multiple downstream signals through the PI 3-kinase and ERK1/2 cascades (26). Both pathways are regulated by Irs1 or Irs2, and both Irs proteins are expressed in β cells; however the Irs2 branch appears to be most important in mice (27). Irs proteins contain similar but not identical pleckstrin homology (PH) and phosphotyrosine-binding domains at their NH_2-terminus that mediate coupling with activated membrane receptors. Cell-based experiments have not revealed a specificity that could explain the functional selectivity observed for Irs2 in β cells. However Irs2 interacts with the activated kinase regulatory loop of the insulin receptor, revealing a unique mechanism for coupling that is not shared with Irs1 (28). By contrast, the PH domain in Irs1 binds to PHIP, a protein that might contribute unique specificity (29).

The Irs2 → PI 3-kinase cascade controls many downstream elements, including Akt/protein kinase B, p70^{s6k}, BAD/Bcl2, and the Foxo subfamily of transcription factors (30). Recently Akt1 and p70^{s6k} were shown to promote β cell growth and function in mice, supporting the importance of signaling components regulated in β cells by the Irs2 → PI 3-kinase cascade (31, 32). One way that Akt promotes survival of cells is by phosphorylation of BAD, which promotes the dissociation of active Bcl2 (33). Since apoptosis of β cells might be crucial at several points during the onset of type 1 or type 2 diabetes, BAD phosphorylation stimulated by the Irs2 → PI 3-kinase → Akt cascade might be an important signal for β cell survival (34, 35). Previous reports show that *Irs2*$^{-/-}$ β cells undergo increased apoptosis at weaning, which might contribute to the early loss of β cell mass (4); however, we did not investigate the effect of *Pdx1* expression on apoptosis during weaning and did not detect apoptosis at later ages.

Pdx1 expression and function might be entirely independent of Irs2 signaling and reduced in *Irs2*$^{-/-}$ islets owing to β cell progressive failure. In this case, transgenic expression of *Pdx1* could overcome the negative effects of the *Irs2* knockout by strongly promoting the expression of genes that are essential for β cell function (10, 11). However, our results are consistent with the hypothesis that Irs2-signaling directly regulates expression and function of Pdx1. Preliminary evidence suggests that *Pdx1* expression in β cells is repressed through nuclear localization of the transcription factor Foxo1 (also called FKHR) (36). Foxo1 is ordinarily exported from the nucleus and retained in the cytosol by Akt-mediated phosphorylation (33). However, Foxo1 is largely nuclear in

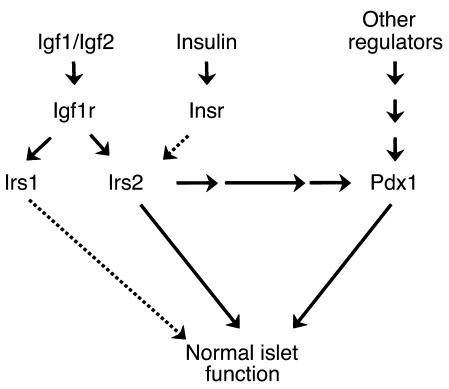

Figure 7
Proposed model of the multiple pathways linking Irs2 signaling and Pdx1 action in β cells.

Irs2$^{-/-}$ β cells, consistent with the relative inactivity of the PI 3-kinase → Akt cascade (36). Interestingly, disruption of a single *Foxo1* allele reduces the level of nuclear Foxo1 in *Irs2*$^{-/-}$ β cells, which increases *Pdx1* expression and restores sufficient β cell function to normalize glucose homeostasis (36). More details about the integration of β cell transcription factors with the Irs2 branch of the insulin/Igf signaling cascade might reveal new strategies to promote β cell growth and function in both type 1 and type 2 diabetes (Figure 7).

PDX1 is critical for the development of the pancreas, and pancreas agenesis occurs upon the complete disruption of *PDX1* in mice and humans (7, 19). Although Pdx1 levels are low in *Irs2*$^{-/-}$ mice and lower in the *Irs2*$^{-/-}$*Pdx1*$^{+/-}$ mice, the exocrine pancreas develops normally unless *Pdx1* is completely disrupted. During early development, *Pdx1* expression might be largely independent of Irs2 signaling, whereas after birth *Pdx1* might be regulated by Irs2-dependent and -independent mechanisms (Figure 7). The hepatocyte nuclear factor Hnf3β promotes expression of *Pdx1*, so its reduced expression in *Irs2*$^{-/-}$ β cells might contribute to their failure (6, 37). However, genetic disruption of *Hnf3β* in β cells causes hyperinsulinemia at birth, possibly owing to the reduced expression of the ATP-sensitive potassium channel in β cells (38). So *Hnf3β* might not be an essential element for *Pdx1*-mediated β cell function. The interaction of these related transcription factors and their integration with receptor signaling are an important direction of future work.

At the physiological level, the relation between *Irs2* signaling and Pdx1 function is supported by the common development of diabetes between 10 and 15 weeks of age in *Irs2*$^{-/-}$ mice, or in mice lacking β cell *Pdx1* (2, 13). The progressive dysfunction of β cells specifically lacking *Pdx1* is related to the reduced expression of genes that promote glucose-sensitive insulin secretion, including proinsulin, glucose transporter-2, glucokinase, and components of the FGF receptor signaling system that control prohormone convertase 1/3 expression (10–12). Consistent with the relation between Irs2 and Pdx1, these gene products were always detected weakly by immunostaining *Irs2*$^{-/-}$ β cells, whereas they were strongly detected in *Irs2*$^{-/-}$*Pdx1*^{tg} β cells (data not shown). These preliminary results are consistent with the hypothesis that *Pdx1* mediates many of the effects of Irs2 on β cell function.

Irs2$^{-/-}$*Pdx1*$^{+/-}$ and *Irs2*$^{-/-}$*Pdx1*$^{-/-}$ mice were hyperglycemic at birth, owing apparently to the near absence of β cells or the absence of a pancreas, respectively. However, *Pdx1*$^{-/-}$ mice were euglycemic until P4, suggesting that extrapancreatic ligands might regulate neonatal glucose homeostasis through Irs2. The importance of Irs2 at this stage is consistent with its role in hepatocytes to mediate the inhibitory effects of insulin on gluconeogenesis (3).

Based on our results we conclude that the Irs2 branch of the insulin/Igf signaling pathway is important for the growth and function of murine β cells. Many of the effects of Irs2, including β cell growth and function are closely associated with *Pdx1* expression. Our results are consistent with a direct link between Irs2 and Pdx1 expression and function; however, additional experiments using cell-based strategies are necessary to prove this hypothesis. Our experiments provide a starting point to understand the relation between β cell failure and states of chronic insulin resistance. Moreover, dysregulation of *Pdx1* by genetic or functional mechanisms might be one of the common links between early-onset (MODY) and ordinary type 2 diabetes.

Acknowledgments

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